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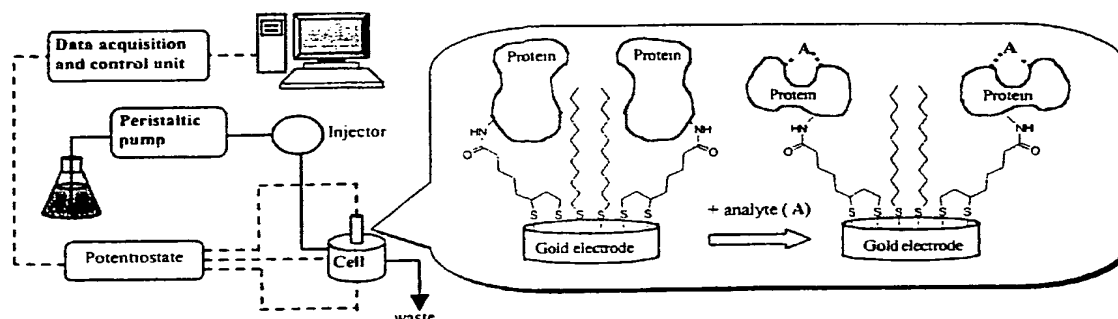
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(54) Title: **DEVICE FOR MONITORING ANALYTES AT SUB-MICROMOLAR CONCENTRATIONS**



(57) Abstract: The invention provides a device for detecting sub-micromolar concentrations of substances that interact with proteins having nucleic acid transcription regulatory activity. The device comprises an electrode having a surface bound by proteins having nucleic acid transcription regulatory activity. Interaction of the protein with a substance induces a conformational change in the protein, resulting in generation of a signal by the device. The invention also provides methods of detecting substances that interact with proteins having nucleic acid transcription regulatory activity as well as methods of identifying active sites of proteins having nucleic acid transcription regulatory activity.

Device for Monitoring Analytes at Sub-Micromolar Concentrations

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to devices for detection of interactions between proteins and their substrates. In particular, the invention relates to biosensors that are capable of detecting interactions between proteins involved in regulation of transcription of nucleic acids, and other proteins or small molecules (*i.e.*, substances) that affect the activity of such proteins. It also relates in particular to methods for detecting substances that interact with proteins involved in regulation of transcription of nucleic acids. In addition, it relates to systems for detecting substances that interact with proteins involved in regulation of transcription of nucleic acids.

Description of Related Art

Selective detection of target analytes present in a complex sample matrix is of great importance in the fields of medicine, the environment, and industry. Biosensors are very promising candidates for this purpose. Among the various types of biosensors available, the amperometric and potentiometric types have been the most studied in the field. However, results have shown that these types of biosensors typically display a detection limit of at most 10^{-8} M. Thus, they are not suitable for monitoring analytes in low (sub-nanomolar) concentration ranges.

Recently, a very sensitive capacitive transducer was reported by Berggren et al. (ref. 1) This transducer is capable of monitoring antigens (*e.g.*, human chorionic gonadotropin; HCG) in the pg/ml range. The same type of transducer was also successfully used to monitor heavy metal ions in the femtomolar range using engineered proteins (ref. 2). Thus, this type of transducer seems to be sufficiently sensitive to monitor conformational changes that occur upon binding of a target analyte/substrate to its cognate receptor (*e.g.*, protein, DNA).

Specifically, Berggren et al. (ref. 1) discloses the use of antibodies immobilized on gold surfaces via self-assembled coupling reactions. During production of the transducer, the coupler and the antibody form an insulating layer on

a gold electrode, which is part of the transducer device. The sensitivity of the resulting electrodes was found to be exceptional on the tested applications. Moreover, the interaction between the antibody and the antigen could be sensed directly, making the use of labels unnecessary.

Recognizing the possibility of using the technique and device of Berggren et al. for selective and sensitive heavy metal detection, Bontidean et al. (ref. 2) immobilized fused proteins on the surface of the gold electrodes. Like Berggren et al., Bontidean et al. obtained very promising results with such a system. In a related publication, Bontidean et al. showed that heavy metal ions could be monitored throughout a large range, from femto- to micro-molar concentrations (ref. 23). Special metal-binding proteins were immobilized as biological recognition elements. Included among the proteins studied were the broad selectivity metallothionein-cyanobacterial metal binding protein SmtA, which was fused to GST (glutathione-S-transferase) and denoted GST-SmtA, and the more selective mercury-binding regulatory proteins MerR and MerP (all overexpressed in *E. coli*). The conformational change occurring upon binding of the heavy metal ions to proteins could be reproducibly monitored even at very low analyte concentrations (ref. 2).

Recently, phytochelatins have been immobilized on the same type of capacitive transducer and the results obtained showed that heavy metal ions could be detected at very low (sub-nanomolar) concentration ranges (unpublished results of inventor).

Other publications reporting detection of metal ions using conformational changes in proteins bound to electrodes include references Brown et al. (ref. 24), Corbisier et al. (ref. 25), and Bontidean et al. (ref. 26).

The repressor proteins represent a group of bioselective proteins with high affinity for their cognate ligand (inducer) (refs. 4, 5). Many repressor proteins undergo a dramatic conformational change upon binding of the inducer molecule. For example, sequence specific interactions of proteins with DNA are central to aspects of the utilization of genetic information in organisms. The lactose (*lac*) repressor of *Escherichia coli* (*E. coli*) has served as a paradigm for such interactions. Indeed, the

lac repressor served as a paradigm even before the chemical structure of the interacting partners was clear (ref. 4).

The binding of the *lac* repressor to the *lac* operator is a good example of a specific and tight interaction between protein and DNA. In order for the organism to utilize lactose as a carbon and energy source, site-specific DNA recognition of the *lac* repressor must be interrupted by lactose, or a lactose derivative, in the environment. Thus, modulation of *lac* repressor/operator interaction is fundamental to induction of the *lac* operon, and is accomplished by binding of specific sugars (inducers) to the repressor protein.

The *lac* repressor protein recognizes the *lac* operator, which comprises a particular, specific region of the chromosome of *E. coli*. The repressor binds to the operator tightly, with a dissociation constant of 10^{-11} to 10^{-13} M (ref. 5). The *lac* repressor is a member of a large family of related proteins sharing significant similarity with the galactose (*gal*) repressor and a number of other repressor proteins and periplasmic sugar-binding proteins found both in *E. coli* and other prokaryotes. The lactose repressor is a tetrameric protein with a molecular mass of 150 kDa (refs. 6, 7) with binding sites for four inducer molecules (refs. 8, 9) and two operator sequences (refs. 10, 11, 12). The protein binds with high affinity to operator sequences, and with significantly lower affinity to nonspecific sequences (ref. 4). Sugars have been found to both destabilize and stabilize *lac* repressor-operator complexes. The former are known as inducers, while the latter are termed anti-inducers. The majority of sugars that bind to the *lac* repressor are inducers, and are galactose derivatives, like isopropyl- β -D-thiogalactoside (IPTG), or 1,6-allolactose. However, there are also neutral compounds like o-nitrophenyl- β -D-galactoside (ref. 13) and anti-inducers, for example, lactose (refs 13, 14) and o-nitrophenylfucoside (ONPF) (ref. 15).

The complexes formed by the *lac* repressor with operator DNA and inducers have been extensively studied by a number of techniques, including spectroscopy, for example NMR (ref. 16); crystallography (ref. 17); circular dichroism (ref. 18); electrochemical methods, like gel electrophoresis mobility shift assays (ref. 19); surface plasmon resonance (ref. 20), or simply by capture of repressor-operator

complexes into nitrocellulose filters (ref. 21). The study of other transcriptional regulatory systems has likewise been studied using various spectroscopic, biochemical, crystallographic, and electrochemical techniques.

However, the methods and devices used to characterize proteins having transcriptional regulating activities are characterized by fairly low sensitivity. Therefore, new devices and methods for characterizing such proteins, and the substances that bind to them, is needed in the art.

SUMMARY OF THE INVENTION

The present invention addresses the needs of the art by providing devices and methods for detecting interaction between 1) a protein having transcriptional regulating activity and/or other binding functionalities and 2) a substance that binds to, or otherwise interacts with, the protein. The devices and methods utilize conformational changes in the protein upon interaction with the substance. The devices and methods of the invention provide high sensitivity (down to the femtomolar or lower range), accuracy, reproducibility, and ease of use. Together, the devices and methods of the invention comprise part or all of a system for detecting substances that interact with proteins involved in regulation of transcription of nucleic acids.

In a first aspect, the invention provides a device. In general, the device comprises an electrode that has bound to it a protein that has transcriptional regulatory activity. The device can 1) bind, either reversibly or irreversibly, a protein that has transcriptional regulatory activity or other binding functionalities, 2) detect a change in conformation of a bound protein that has transcriptional regulatory activity or other binding functionalities, 3) produce a signal upon detection of a change in conformation of a bound protein that has transcriptional regulatory activity or other binding functionalities, where the signal produced is either detectable using the device or is transferable from the device to another device that can display the signal.

Nucleic acids according to the invention are either DNA or RNA (or hybrids of DNA and RNA). Nucleic acids can be single-stranded or double-stranded. A

protein according to the invention is any protein, including those from eukaryotic cells and/or prokaryotic cells, those recombinantly produced, and those that are partially or totally chemically synthesized.

Proteins according to the invention include polypeptides and peptides; thus, they include fragments of proteins having transcriptional regulatory activity.

By transcriptional regulatory activity, it is meant an activity that affects, either positively or negatively, either directly or indirectly, the level or rate of transcription of a nucleic acid. The activity can be an activity that is present *in vivo*, that is, in a living cell or under physiological conditions, or an activity that is present *in vitro* under pre-defined conditions that can be, but are not necessarily, similar or substantially or totally identical to conditions in which the protein functions in its natural environment (*i.e.*, *in vivo*).

Proteins with other binding functionalities are proteins that do not regulate the process of transcription of nucleic acids. Thus, they can be heavy metal binding proteins, chaperonins or other proteins involved in proper folding, processing, etc. of proteins, and enzymes.

Binding or interacting means physical interaction of any kind, including any kind of interaction that can be considered an interaction via electrostatic forces, van der Waals forces, hydrogen bonding, hydrophobic interactions, ionic bonding, and/or covalent bonding. Binding or interaction occurs under the conditions of the assay milieu. Therefore, the systems of the invention can provide information about binding of a protein and a substance under many different conditions, some of which can be, but are not necessarily, similar or substantially identical to, physiological conditions.

In a second aspect, the invention provides a method for detecting the presence of a substance of interest in a sample suspected of containing the substance. In general, the method comprises: providing a device comprising an electrode that has bound to it a protein that has transcriptional regulatory activity; exposing the device to a sample suspected of containing the substance of interest under conditions where specific interaction of the substance with the protein bound to the electrode can occur; and determining whether the protein has interacted with the substance of interest. If an interaction has occurred, a signal produced by the electrode will be generated, and

can be detected. Presence of a signal indicates the presence of the substance of interest in the sample.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a general scheme for a system according to the invention.

Figure 2 shows a graph indicating that conformational changes in a protein having transcription regulatory activity can be detected upon interaction with various substances with an exemplary device and method according to the invention.

Figure 3 shows a graph indicating the effect of the conformation and size of a representative analyte upon the signal generated by an exemplary system according to the invention. The graph indicates that analyte conformation can have an effect on the sensitivity of systems according to the invention and that analyte size can have an effect on the sensitivity of systems according to the invention.

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

In a first aspect, the invention provides a device comprising an electrode that has bound to it a protein that has transcriptional regulatory activity. The electrode can be any physical substance, or composition of substances, that can conduct an electrical signal. In embodiments, the electrode is a conducting material. In embodiments, the electrode is a non-conducting material, such as plastic, that is covered by a conducting material. Thus, the electrode according to the invention can 1) bind, either reversibly or irreversibly, a protein that has transcriptional regulatory activity or other binding functionalities, and 2) translate a change in conformation of a bound protein that has transcriptional regulatory activity or other binding functionalities into a measurable signal, for example a change in capacitance, upon binding of the protein to a substance. Electrodes that satisfy these requirements are known in the art, and any suitable electrode or similar device under a different name, can be used, as long as it provides the sensitivity according to the present invention. In embodiments, the electrode is identical or substantially similar to the capacity affinity sensor described in WO 99/14596. Construction of such an electrode is disclosed in detail in that document. Briefly, according to the present invention, the method for constructing the

electrode comprises: 1) covering a solid element with a noble metal, and 2) covering the noble metal surface with a layer of a self-assembling monolayer-forming molecules comprising coupling groups.

The electrode comprises a solid element that is either conducting or non-conducting. In embodiments where the solid element is non-conducting, the solid element is covered by a layer of conducting material, such as a layer of noble metal. In embodiments, the electrode comprises a solid element that is plastic. In embodiments, the electrode comprises a solid element that comprises silicon. In certain embodiments, the solid element is covered by a layer of gold. The gold can be applied to the solid element using various techniques, including, but not limited to, sputtering of the gold onto the surface of the solid element. The sputtered gold layer can be relatively thick or thin, as long as it is thick enough to allow formation of a monolayer on it.

According to the present invention, the noble metal can be gold, silver, copper, platinum, or palladium. In embodiments, it is gold.

According to the present invention, the self-assembling monolayer-forming molecules are molecules that are capable of forming a self-assembling monolayer on a noble metal. Such molecules can, but do not necessarily, comprise at least one thiol, sulfide, or disulfide group. For example, the monolayer can be an alkyl-thiol layer. Optionally, they can comprise an activated (cross-linking) group to aid in binding of the protein having transcriptional regulatory activity. Thus, in embodiments, the self-assembling monolayer-forming molecules are capable of associating, without further chemical modification, with at least one protein having nucleic acid transcriptional regulatory activity. In other embodiments, the self-assembling monolayer-forming molecules are treated to activate them before they are layered onto the metal. Activation aids in association of the protein having transcriptional regulating activity to the monolayer-covered electrode. In embodiments, the self-assembling monolayer-forming molecules are molecules that can self assemble on gold or other noble metals and that have a functionalized group.

Examples of self-assembling monolayer-forming molecules include, but are not limited to, thiocetic acid and cysteamine. Compounds for activation of the

monolayer, that is, for cross-linking the monomer layer to the proteins having transcription regulatory activity or other binding functionalities, are known to those of skill in the art. An example of such an activator is 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide. Other examples include, but are not limited to, glutaraldehyde and polyethylene glycol diglycidyl ether.

Construction of the device according to the invention includes binding affinity molecules (*i.e.*, proteins with nucleic acid transcriptional regulating activity or other binding functionalities) to the monolayer of the electrode, followed by covering any remaining free spots on the noble metal surface of the electrode (*i.e.*, any spots that are not covered by the monolayer) by a second self-assembling monolayer-forming molecule, which can be the same or different than the first monolayer-forming molecule.

In embodiments, a thiol monolayer is self-assembled on an electrode having a gold surface layer. The monolayer is activated with carbodiimide. Subsequently, a protein having nucleic acid transcription regulatory activity or other binding functionalities is bound to the monolayer. Any free spot on the electrode not covered by gold, thiol monolayer, or protein is then covered in order to eliminate bias/false signals from any other electrochemically active substance present in the sample to be assayed. In embodiments, a long-chain polymer is used to cover the electrode at this point.

The proteins bound to the monolayer of the electrode can be bound irreversibly or reversibly. Reversible binding is defined as binding that can be reversed without the need to break covalent bonds between the protein and the monolayer or within the protein molecules or the monolayer molecules, or otherwise irreversibly modify or destroy the secondary or tertiary structure of the monolayer or the protein. Irreversible binding requires that, in order to reverse the binding, covalent bonds must be broken, or the secondary or tertiary structure of the protein or the monolayer must be destroyed or modified to an extent that reformation of the original structure would require at least one covalent modification of the protein or monolayer.

When bound irreversibly, for example through covalent attachment of the protein to the molecules making up the monolayer, the device can be used under a

wide range of conditions without the fear that the protein will be lost from the device. For example, the device can be used in compositions having a very high or a very low pH (*e.g.*, greater than 10 or less than 3), a very high or very low salt concentration (*e.g.*, less than 1 mM or greater than 1 M total salt), or high heat. When bound irreversibly, the device can also be cleaned repeatedly, and thus reused multiple times. Although destruction or modification of one or both of the protein and/or the monolayer will occur upon removal of the protein from the monolayer, it is possible to remove the protein from the monolayer without damaging the monolayer or the underlying metal or solid element. That is, removal may alter only the bound protein or changes to the monolayer can be easily reversed by chemical treatment. Thus, in embodiments, the device can be "regenerated" by removal of the protein from the monolayer, and subsequent binding of new protein. In embodiments, other substances, such as contaminants, bound to the monolayer can be removed before, during, or after removal of the protein from the monolayer. In certain embodiments where the device is regenerated, the same protein is bound to the monolayer. In other embodiments, after removal and optional cleaning of the monolayer, a new, different protein is bound to the monolayer. In this way, a single device can be used to assay multiple different proteins having transcription regulatory activity. Where necessary or desirable, exposed metal of the device can be re-covered with monolayer before, during, or after subsequent binding of new protein to the monolayer. Thus, when the protein is bound irreversibly, the device provides a convenient, rapid way of repairing the device or altering the specificity of the device. Other advantages will be apparent to those of skill in the art, and need not be listed here.

Reversible binding of the monolayer and the protein having transcription regulatory activity can permit the device to be easily "regenerated" multiple times without the loss of sensitivity or physical integrity of the device. That is, upon loss of sensitivity, or for any other reason, the protein can be removed from the monolayer, and new protein can be bound to the monolayer. In embodiments, other substances, such as contaminants, that are bound to the monolayer can be removed as well before, during, or after removal and rebinding of protein to the monolayer. In certain embodiments where the device is regenerated, the same protein is bound to the

monolayer. In other embodiments, after removal and optional cleaning of the monolayer, a new, different protein is bound to the monolayer. In this way, the device can be used to assay multiple different proteins having transcription regulatory activity. Where necessary or desirable, exposed metal of the device can be re-covered with monolayer before, during, or after subsequent binding of new protein to the monolayer. Thus, when the protein is bound reversibly, the device provides a convenient, rapid way of repairing the device or altering the specificity of the device. As with embodiments where the protein is bound irreversibly, in embodiments where the protein is bound reversibly, the device can be used in a variety of compositions. The type of compositions that the device can be used in will depend on the strength and nature of binding between the protein and monolayer. Of course, in embodiments where the protein is reversibly bound to the monolayer, the device can be cleaned repeated using an appropriate cleaning composition, which will minimize loss of the protein from the monolayer. In embodiments where cleaning removes part, but not all or substantially all of the protein from the monolayer, new protein can be bound to the monolayer after cleaning, as necessary or desired. Other advantages will be apparent to those of skill in the art, and need not be listed here.

Accordingly, the device can be designed for single use or multiple use. That is, it can be used a single time and discarded, or it can be used multiple times, and with multiple different proteins if desired, before discarding. Accordingly, the present invention provides a disposable device for detecting the presence of substances that interact with a protein having nucleic acid transcription regulating activity. Thus, the present device is highly versatile and provides a convenient means for detecting the presence of multiple substances in various different milieus.

The signal detected by the device of the present device is a signal generated by a change, even a slight change, in conformation of the protein bound to the monolayer. As discussed above, the electrode comprises a surface that is covered with a monolayer of, for example, long-chain alkenethiols. Capacitive transduction typically relies on a double layer of material between a metal electrode and the solution being assayed, resulting in a double-layer capacitance as well as a Faradaic current, which gives rise to a Faradaic background current for electrochemical measurement.

Although the present device does not comprise a double-layer capacitance in the usual meaning, it still is capable of transmitting a capacitance that can be accurately and reproducibly measured.

The capacitance change is measured by applying a fast potentiostatic pulse to the device and evaluating the resulting current transients. As discussed below, in embodiments, the device of the invention further comprises other components that receive, evaluate, and/or display the signal produced by the device of the invention. In those embodiments, the other components can include a computer to control analysis of the signal.

According to the present invention, nucleic acid transcription regulatory activity is defined as an activity that affects, either positively or negatively, either directly or indirectly, the level or rate of transcription of a nucleic acid. The activity can be an activity that is present *in vivo*, that is, in a living cell or under physiological conditions, or an activity that is present *in vitro* under pre-defined conditions that can be, but are not necessarily, similar or substantially or totally identical to conditions in which the protein functions in its natural environment (*i.e.*, *in vivo*). Thus, the device of the present invention can be used to identify one or more natural substrate, binding partner, agonist, or antagonist for the protein having nucleic acid transcription regulatory activity that is being investigated. Alternatively, the device of the present invention can be used to identify one or more substrate, binding partner, agonist, or antagonist for the protein having nucleic acid transcription regulatory activity that is being investigated, where the substrate, etc. interacts with the under conditions that are not substantially similar to the environment in which the protein naturally exists. Accordingly, in embodiments, the device can be used to identify substances that might be similar, but not identical, in activity to a substrate that naturally binds to the protein under investigation. Such substances can provide insights into the physical nature of the natural substrate, etc., and can be used, for example, to design derivatives of the natural substrate that can bind to the protein of interest *in vivo*, but have altered biological or biochemical properties (*e.g.*, longer or shorter half-life *in vivo*, higher or lower binding affinity for the protein, greater solubility, lower toxicity).

Proteins having nucleic acid transcription regulatory activities are well known to those of skill in the art. The proteins may be prokaryotic, eukaryotic, or viral. Any of these proteins can be used in the device, method, and systems of the invention.

Exemplary proteins include, but are not limited to, regulatory proteins involved in bacterial metabolism, such as regulatory proteins involved in utilization of carbon and energy sources, synthesis of cell superstructures (cell walls, membranes, genomes, etc.), and catabolism and anabolism of amino acids and nucleotides. Two specific examples are the bacterial *lac* repressor and the bacterial *gal* repressor. Exemplary proteins also include, but are not limited to, regulatory proteins involved in bacterial phase variation or regulation of other pathogenic characteristics.

Other exemplary proteins include, but are not limited to, proteins involved in viral infection, replication, and evasion of host immune defense systems.

Other exemplary proteins include, but are not limited to, proteins involved in regulation of eukaryotic gene expression, such as expression of parasite genes, animal genes, and human genes. Examples include, but are not limited to, proteins involved in regulation of animal, including mammalian and thus human, immunity and immune system responses. Examples also include, but are not limited to, proteins involved in cancer or other tumors, cell death (e.g., apoptosis), and genetic diseases (i.e., diseases showing a link to a particular gene or collection of genes).

The device provides extremely high sensitivity for detecting interactions of the protein bound to the surface and substances that react with the protein. By detecting conformational changes in the protein bound to its surface, the device can detect the presence of a substance in the original sample at concentrations in the micromolar range (i.e., 1-999 micromolar). In embodiments, the device can detect the presence of a substance at a concentration of less than 1 micromolar. Indeed, the device can detect interactions at concentrations of a substance at less than 1 nanomolar. For example, sensitivities can be as low as the single-digit femtomolar range or lower.

In embodiments, the device can detect interactions at a concentration of substance in the original sample of between 1 nanomolar and 10 micromolar.

In embodiments, the device can detect interactions at a concentration of a substance in the original sample between 1 and 999 nanomolar. For example, in

embodiments, the device can detect interactions where the substance is present in the original sample between 1 and 500 nanomolar. In embodiments, the device can detect interactions where the substance is present between 1 and 250 nanomolar. In embodiments, the device can detect interactions where the substance is present between 1 and 100 nanomolar. In embodiments, the device can detect interactions where the substance is present between 1 and 50 nanomolar. In embodiments, the device can detect interactions where the substance is present between 1 and 10 nanomolar.

In other embodiments, the device can detect interactions at a concentration of substance in the original sample of less than 1 nanomolar. For example, in embodiments, the device can detect interactions at a concentration of substance in the original sample between 1 and 999 picomolar. In embodiments, the device can detect interactions where the substance is present in the original sample between 1 and 500 picomolar. In embodiments, the device can detect interactions where the substance is present between 1 and 250 picomolar. In embodiments, the device can detect interactions where the substance is present between 1 and 100 picomolar. In embodiments, the device can detect interactions where the substance is present between 1 and 50 picomolar. In embodiments, the device can detect interactions where the substance is present between 1 and 10 picomolar.

In other embodiments, the device can detect interactions at a concentration of substance in the original sample of less than 1 picomolar. For example, in embodiments, the device can detect interactions at a concentration of substance in the original sample between 1 and 999 femtomolar. In embodiments, the device can detect interactions where the substance is present in the original sample between 900 and 999 femtomolar. In other embodiments, the device can detect interactions where the substance is present between 750 and 999 femtomolar. In yet other embodiments, the device can detect interactions where the substance is present between 500 and 999 femtomolar. In still other embodiments, the device can detect interactions where the substance is present between 250 and 999 femtomolar, such as between 250 and 750 femtomolar or between 250 and 500 femtomolar. In further embodiments, the device can detect interactions where the substance is present between 100 and 999

femtomolar, such as between 100 and 750 femtomolar, between 100 and 500 femtomolar, or between 100 and 250 femtomolar. In some embodiments, the device can detect interactions where the substance is present between 1 and 250 femtomolar, such as between 1 and 100 femtomolar, 1 and 50 femtomolar, or 1 and 10 femtomolar. Other exemplary ranges include between 50 and 750 femtomolar, between 50 and 500 femtomolar, and between 50 and 750 femtomolar.

The sensitivity of the present device is such that it can be used to detect as low as 1-100 molecules in the sample. For example, it can be used to detect from 1-50, 1-100, 1-250, 1-500, or 1-1000 molecules of a substance of interest.

In a second aspect, the invention provides a method for detecting an interaction between a protein having nucleic acid transcription regulatory activity or other binding functionalities and a substance. The method achieves this goal by sensing conformational changes in the protein. In general, the method comprises: providing a device comprising an electrode that has bound to it a protein that has transcriptional regulatory activity or other binding functionalities; exposing the device to a sample suspected of containing the substance of interest under conditions where specific interaction of the substance with the protein bound to the electrode can occur; and determining whether the protein has interacted with the substance of interest. If an interaction has occurred, a signal will be generated, and can be detected. Presence of a signal or a change in the signal, indicates the presence of the substance of interest in the sample.

According to the method of the invention, a sample suspected of containing the substance that interacts with the protein is exposed to the device under conditions such that physical contact between the substance and the protein can occur. Such conditions are widely known to those of skill in the art or can be determined without undue or excessive experimentation. Thus, they need not be detailed here. Upon contact of the substance and the protein, there will be a change in the capacitance or impedance of the device. The capacitance or impedance change takes place between the solution in which the substance is present and the metal surface of the device. The changes are essentially electrochemical perturbations that occur in the device. Faradaic reactions that would occur between substances in the sample with the metal

of the device, as well as background currents, are blocked by the monolayer and protein on the surface of the device, in effect creating an insulative layer on the metal. Thus, the changes are indicative of changes in the protein itself, such as conformational changes.

A signal corresponding to the change in capacitance or impedance is generated by the device and is directly related to the interaction of the protein and a substance in the sample being tested. The signal is either immediately detectable by a human, or is further processed by additional components of the device (or the system, as detailed below) to provide a detectable signal. In embodiments, at least one measurement of the signal is taken. In embodiments, the signal or the measurement of the signal, is recorded.

In embodiments, the changes in capacitance or impedance are potentiostatic steps or pulses. These steps or pulses generate current transients from which the capacitance or impedance, and the change in capacitance or impedance, can be determined. In embodiments, the changes in capacitance or impedance are amperometric steps or pulses. In these embodiments, the capacitance or impedance, and the change in capacitance or impedance, can be determined by determining the change in potential.

In the method of the invention, if a substance is present that specifically interacts with the protein on the surface of the device, a change in capacitance or impedance occurs, and a conclusion can be drawn that such a substance is present. If such a substance is not present, no significant change will occur. Specificity of binding can be adjusted by adjusting the assay conditions. Thus, if no specific binding occurs under a given assay condition, the conditions can be changed, and the assay re-run. Accordingly, the method of the invention can provide information on the optimal conditions for binding of a substance and a protein. Often, binding using the method and device of the invention indicates binding that is relevant to an *in vivo* system of interest. However, at other times, it will be indicative of specific binding under pre-chosen conditions, and thus might not be physiologically relevant. However, although the results might not be physiologically relevant, they can provide information that is useful for other purposes, for example to identify optimal conditions for assaying

substances that interact with proteins under industrial or other non-natural settings. The information can also be used in identifying portions of proteins that are important for binding to substances or for identifying proteins, or portions thereof, that interact with a given substance, where determination of optimal interaction is not a high priority.

In embodiments, the method is performed in a batch cell. In these embodiments, a known volume of sample is combined with a known volume of a conducting liquid in a cuvette in a batch cell. The device is exposed to the combination, and interaction between the protein on the surface of the device and a substance in the combination is assayed. In embodiment, the method is performed in a flow cell. In these embodiments, a known volume of sample is injected into a conducting carrier liquid, which is flowing past the device at a pre-set flow rate.

In embodiments, the sample is provided as a solution comprising the substance of interest. In embodiments, the solution comprises water as the solvent. In embodiments, the solution comprises an organic compound as the solvent. In embodiments, the solution comprises a mixture of water and one or more organic compounds as the solvent. Suitable organic solvents are known to those of skill in the art or can be identified without undue or excessive experimentation, and thus need not be detailed here.

The substance to be assayed can be any substance that interacts with a protein having nucleic acid transcription regulatory activity or other binding functionalities. Thus, the substance can be anything from a simple salt or organic compound to a complex polymer or macromolecule. It is known that small molecules, such as salts, sugars and their derivatives, nucleotides, nucleosides, co-factors, and the like, bind to various proteins and alter the activity of the protein. Included among these proteins are proteins having nucleic acid transcription regulatory activity. Also included among these proteins are proteins having other binding functionalities.

Examples of substances that can be detected include, but are not limited to, elements and salts, such as the elements, and salts thereof, of calcium, sodium, magnesium, manganese, iron, molybdenum, and phosphorus. Other examples of substances include, but are not limited to, small molecules, such as second

messengers, nucleotides or nucleosides, amino acids, fatty acids, steroids and other aromatic group-containing compounds, and sugars. Yet other examples include, but are not limited to, polymers, such as peptides, polypeptides, proteins, nucleic acids, polysaccharides, lipids, and copolymers or derivatives thereof (*e.g.*, lipopolysaccharides, glycoproteins, etc.). One group of substances that can be detected according to the present method are drugs, including both pharmaceuticals (*i.e.*, those that have therapeutic value) and non-pharmaceuticals (those with little or no significant therapeutic value). Furthermore, a substance according to the invention can be a living cell or a virus, including prokaryotic cells, eukaryotic cells, animal viruses, and phages (bacterial viruses). Examples of eukaryotic cells include, but are not limited to, cancer or other tumor cells, cells associated with a disease or degenerative condition (*e.g.*, cells associated with Alzheimer's disease, Parkinson's disease, Huntington's disease, multiple sclerosis, lymphomas, etc. Accordingly, the method of the invention can detect cells or viruses responsible or associated with diseases or disorders, such as cancer cells, viruses, or pathogenic bacteria.

Substances according to the invention also include compounds, etc. that mimic known drugs or other substances with biological activity. Such mimics can be useful not only for understanding the activity of the drug, narcotics, etc. that they resemble, but also as substitutes for the drug, narcotic, etc. Such substitutes can have altered biological activities and properties, such as increased potency, increased solubility, reduced toxicity, reduced potential for addiction, and the like.

Other substances according to the invention are pesticides, fungicides, herbicides, poisons, and similar toxic substances. Detection of the presence of pesticides or other poisonous or toxic substances in the environment and in food is an important aspect of maintaining health and safety.

Further substances include explosives, such as nitroaromatics. Currently, nitroaromatics, such as those present in buried, unexploded arms, can be detected using immunological methods. However, the methods of the present invention will enable one to detect much lower levels of explosive compounds, such as nitroaromatics, and thus will enable practitioners to identify potentially dangerous sites that were previously unidentifiable. For example, regulatory proteins from

bacteria capable of metabolizing such compounds can be used as probes for samples containing the compounds.

In embodiments, the substance is provided to the method and device as a solid sample. Upon addition to the assay cell, which comprises a pre-set amount of solvent, the substance can be solubilized, suspended, or otherwise made capable of contacting the device. In embodiments, the substance is provided as a component in a liquid sample (a liquid composition). The liquid sample can be a solution, a suspension, a colloid, or any other type of mixture or dispersion comprising the substance. In embodiments, the substance comprises the sole component in the sample (other than the solvent where the sample is a liquid composition). In other embodiments, the substance is one of many substances present in the sample. The concentration and volume of the substance and/or composition can be adjusted as desired to provide sufficient substance and volume to obtain satisfactory results.

In embodiments, the method is a method of detecting the presence of a substance of interest in a sample suspected of containing the substance. In other embodiments, the method is a method of determining the concentration of a substance in a sample suspected of containing the substance. In yet other embodiments, the method is a method of identifying a protein that binds to a substance of interest. In further embodiments, the method is a method of identifying the binding site or the amino acid residues that are important or necessary for binding of the protein to the substance.

In embodiments, the method is a method of determining the concentration of a substance in a sample. In these embodiments, the method of detecting a substance that interacts with a protein having nucleic acid transcription regulatory activity further comprises comparing the signal resulting from exposing the device of the invention to the sample with a standard curve correlating signal to concentration of substance. Preparation of standard curves is a routine matter for those of skill in the art and thus need not be detailed here.

In embodiments, the method is a method of determining the binding constant of a protein for a given substance. In these embodiments, the method of detecting a substance that interacts with a protein having nucleic acid transcription regulatory

activity further comprises exposing the device to multiple samples, each containing different concentrations of a substance known to specifically interact with the protein bound to the surface of the device. By collecting the signal data from each sample, and by providing a sufficient number of sample containing the appropriate concentrations of substance, the binding constant of the protein for the substance can be determined. Likewise, the reaction mechanism of the protein/substance can be determined by including competitors, etc. for the binding site. Techniques for determining binding constants and reaction mechanisms are known to those of skill in the art and need not be detailed here.

In embodiments, the method is a method of identifying the binding site or the amino acid residues that are important or necessary for binding of the protein to the substance. As mentioned above, a protein according to the present invention includes polypeptides and peptides. Thus, it includes fragments of a protein having transcription regulatory activity. Therefore, the device of the invention can be used to identify the binding sites or active sites of various proteins having transcription regulatory activity. That is, a protein having transcription regulatory activity can be dissected into various fragments, bound to a monolayer-covered electrode to make a device according to the invention, then assayed against known substrates, binding partners, etc. of the protein. Doing so can determine the portions of the protein that are required or important for binding of the protein to the substrate, binding partner, etc. For example, it can reveal the domains, specific short amino acid sequences, or individual residues that are important or necessary for binding. In embodiments, the method comprises assaying multiple proteins, each having defined changes in the primary amino acid sequence of a protein of interest (*i.e.*, mutants of the protein of interest), and comparing the signals produced for each mutant. Upon comparison, a conclusion can be drawn as to the importance of each residue under consideration. Further, a conclusion can be drawn as to which residue or group of residues provides the activity desired (*e.g.*, lower or higher affinity for substrate, altered substrate specificity). Accordingly, the present invention provides a method of determining the binding site, the active site, or the regions of a protein having transcription regulatory activity that are involved in binding of the protein to its substrate, binding partner, etc.

In embodiments, the method of the invention is a high-throughput screening method for substances that interact with a protein of interest that has nucleic acid transcription regulatory activity.

In yet a further aspect of the invention, a system is provided for the detection of interaction of a protein that has nucleic acid transcription regulatory activity and a substance. The system comprises the device of the invention and detects a conformational change in the protein upon interaction with the substance. The system comprises, in addition to the device of the invention, some or all of the additional components that can be used to detect, analyze, and display an interaction between a protein that has nucleic acid transcription regulatory activity and a substance. Thus, in essence, the system according to the invention is a multi-component device for detecting an interaction between a protein that has nucleic acid transcription regulatory activity and a substance.

In performing the method of the invention, the device is exposed to a sample that is suspected of containing the substance of interest. In embodiments, the device is inserted into a cell, such as an electrochemical cell. The cell may be any container that is suitable for conducting assays according to the invention. In embodiments, it is a cuvette. In embodiments, it is a flow cell. Typically, the cell contains an auxiliary electrode and a reference electrode. In embodiments, the auxiliary electrode is a platinum foil. The auxiliary electrode can be placed anywhere within the cell. For example, it can be placed symmetrically and opposite to the measuring electrode. In embodiments, the reference electrode is a standard saturated calomel electrode. In embodiments, the reference electrode is a Ag/AgCl electrode. Auxiliary and reference electrodes are known to those of skill in the art, and need not be further detailed here. Typically, the cell has a small volume to minimize dilution of the sample.

In embodiments, the device of the invention is connected to a fast potentiostat. The fast potentiostat can then be connected to a computer, which controls the amount and duration of electrical signal going to each electrode in the cell. Fast potentiostats and computer systems are well-known to those of skill in the art, and need not be detailed here.

Some or all of these additional components can be included, in addition to the device of the invention, in the system of the present invention.

EXAMPLES

The following examples are provided to more fully describe certain embodiments of the present invention. They are not intended to, and should not be construed as, limiting the invention in any way.

Example 1: General Procedures

Device Preparation

Unless otherwise noted, devices used in the examples were prepared as follows:

Gold electrodes are first prepared by covering the solid element with a layer of gold. The gold covering is next polished, treated with ultrasound, and plasma cleaned. Next it is treated with thioctic acid to produce a monolayer covering over the gold layer (see, for example, ref. 1). Then, the thioctic acid self-assembled electrodes are thoroughly washed with pure ethanol, dried, and activated in a 1% solution of 1-(-3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride in dried acetonitrile for 5 hours. After washing with 100 mM potassium phosphate buffer, pH 8, the electrodes are dipped in a solution of the desired protein (approximately 0.05 mg/ml protein) under conditions such that covalent coupling between the protein and the monolayer takes place (for example, at 4°C for 24 hours). The electrodes are then washed with phosphate buffer and immersed in a 10 mM solution of 1-dodecanethiol in ethanol for 20 minutes. A final washing of the protein-modified electrodes with phosphate buffer completes the device preparation.

Capacitance Measurements

Unless otherwise noted, capacitance measurements reported in the examples were made as follows:

Capacitance measurements are taken as described elsewhere (refs. 1, 3). Briefly, the device is inserted as the working electrode in a three or four electrode flow cell with a dead volume of approximately 10 μ l. The flow cell contains a platinum foil and a platinum wire as the auxiliary and the reference electrodes, respectively. The flow cell is shown generally in Figure 1. The electrodes are connected to a fast potentiostat. Samples are injected in to the carrier buffer solution pumped by a peristaltic pump. Measurement are taken by applying a potential pulse of 50 mV and recording the current transients following the potential step according to equation (1):

$$i(t) = u/R_s \exp(-t/R_s C_1) \quad (1)$$

where $i(t)$ is the current at time t , u is the amplitude of the potential pulse applied, R_s is the resistance between the gold and the reference electrodes, C_1 is the total capacitance over the immobilized layer, and t is the time elapsed after the potential pulse was applied.

The current values are collected with a frequency of 50 kHz and the first ten values are used for evaluation of the capacitance. Ten measurements are made with an interval of one second and the capacitance is calculated from the mean. The platinum reference electrode controls the working electrode potential, but it does not have a well-defined potential. However, such an electrode is used to obtain a sharp response in a small dead volume cell. Platinum and Ag/AgCl reference electrodes are therefore compared potentiometrically just before a step is applied. The computer adjusts the working electrode potential so that the potentiostat behaves as having an Ag/AgCl reference controlling the working electrode.

Example 2: Detection of Interaction of Inducers (de-repressors) With the *lac* Repressor

A conformational change in the *lac* repressor structure takes place as a result of binding of a very small amount of inducer. Such a conformational change reduces the affinity of the *lac* repressor for its binding site in the operator region of the lactose

operon region of the host cell's genome. To investigate the ability of a device according to the invention to detect conformational changes in a protein having transcription regulatory activity, a device comprising the *lac* repressor was made and tested. The results are presented in Figure 2.

Biosensors were prepared by immobilizing the *lac* repressor protein on a thioctic acid-modified gold surface by carbodiimide covalent coupling. *Lac* repressor was purified as described previously from *Escherichia coli* BJH8117 (genotype: F⁻ $\Delta(lac-proAB)$ *thi*, *gyrA* (Nal^R), *supE*, λ) (ref. 27).

The device used in this example comprised an electrode of the invention having the *E. coli lac* repressor bound to the monolayer. A three electrode flow cell was used for the assay. An extra Ag/AgCl electrode was placed in the outlet stream to compare the potential with the platinum reference electrode just before measurements were made. In order to apply 50 mV on the working electrode, the computer compared the potential of the Pt with the potential of the Ag/AgCl before applying the pulse on the working electrode. The carrier buffer (10 mM potassium phosphate buffer, pH 7.2) containing 1 mM DTT was degassed before use and pumped at a flow rate of 0.25 ml/min. Samples of 250 μ l were injected in the carrier flow. Measurements were made as described earlier applying a 50 mV potential pulse and recording the current transient. The current values were collected with a frequency of 50 kHz, and the first 10 values were used for the evaluation of capacitance.

The results presented in Figure 2 show that conformational changes in a protein of interest can be easily be detected at concentrations of 1 nanomolar. The sensitivity of the repressor modified electrodes decreased in the order: $S_{ONPG} > S_{IPTG} > S_{\alpha-D \text{ Lactose}}$. The sugar derivative with the highest molecular weight ($M_w=310$), α -D-lactose, gave the lowest signal, while IPTG ($M_w=230$) and ONPG ($M_w=301$) yielded similar responses. All three inducers were detected in the 1 nM - 10 mM concentration range. When saturating amounts of IPTG were injected in the system, the sequential injection of plasmid DNA did not cause any further change in capacitance, suggesting that the operator binding did not occur. The binding of the inducer to the immobilized *lac* repressor protein prevents the repressor binding to the operator.

Example 3: Detection of Interaction of DNA With the *lac* Repressor

As discussed above, it is well known that the *lac* repressor binds to a specific region in the operator of the lactose operon of bacteria. Binding of the *lac* repressor represses transcription of the lactose-utilizing genes. Upon presentation of the cell with a de-repressor such as lactose or derivatives or structural analogs of lactose, binding of the repressor to the operator is blocked, and the genes necessary for metabolism of lactose are transcribed. Blockage of binding to DNA is caused by a conformational change in the *lac* repressor. Thus, it is clear that, in addition to binding to de-repressors, the *lac* repressor also binds to a specific site in the operator of the *lac* operon.

The device of the present invention was used to detect binding of the *lac* repressor to its specific cognate site on DNA. A device comprising the *E. coli lac* repressor was prepared as described above, and DNA samples comprising the *lac* repressor cognate binding sequence were assayed to determine if the device could detect binding of the DNAs. The results are shown in Figure 3.

Plasmid DNA was obtained as follows: plasmid p310 DNA (2455 bp in length), which comprises a 24 bp fragment containing the optimal *lac* promoter, cloned into the *Nhe*I site of plasmid pEE using standard procedures. The resulting plasmid was transformed into competent *E. coli* BMH8117 cells according to standard procedures. The recombinant cells were grown in DYT medium in one liter shaking culture flasks at 37 °C, harvested after 16-20 hours of incubation, washed twice with buffer (0.2 M Tris-HCl, pH 7.2, containing 0.2 M KCl, 10 mM MgCl₂, 5% (v/v) glycerol, 1 mM NaN₃, 0.3 mM dithiothreitol (DTT), and 1 mM PMSF), and stored frozen until use. The frozen recombinant cells were thawed, and plasmid DNA containing the *lac* operator was isolated using the miniprep Qiagen kit method according to manufacturer's instructions. 0.025, 0.05, 0.25, and 0.5 µg of the resulting plasmid were linearized by digesting with *Eco*RI, which cuts only once in the plasmid. Genomic DNA was isolated from *E. coli* using standard protocols.

As can be seen in Figure 3, the signals obtained using linearized plasmid were much greater than those obtained using closed circular, supercoiled, concatameric

plasmid and genomic DNA. Thus, the device according to the invention can provide information regarding not only binding of a substance to a protein, but the accessibility of a protein's binding site for the substance (*e.g.*, its cognate binding partner) as well.

Example 4: Effect of Size of DNA on Binding Affinity of the *lac* Repressor

Previous work by others has shown that binding of the *E. coli lac* repressor to its cognate site in the *lac* operator region was independent of the size of the DNA molecule used to identify binding. To verify this result, DNA molecules of differing sizes were assayed for their ability to interact with a device according to the invention, which comprised the *lac* repressor. The device comprising the *E. coli lac* repressor was prepared as described above.

Briefly, in one sample, the plasmid produced in Example 3, which contains a single optimal *lac* repressor binding site, was digested with *EcoRI* and *HindIII* to create two fragments, one containing the *lac* repressor site (84 bp), and the other containing no *lac* repressor binding site. In a second sample, the plasmid DNA was digested with *EcoRI* alone, resulting in a single linear molecule comprising the entire plasmid (as in Example 3). Genomic *E. coli* DNA according to Example 3 was assayed in a third sample. Each of the two samples was assayed with a device according to the invention comprising the *lac* repressor.

The results are shown in Figure 3. Non-specific genomic DNA gave low signals, showing the ability of the *lac* repressor-modified sensor to distinguish the operator-DNA from the non-operator genomic DNA. Furthermore, the *lac* operator-containing DNA sequences of different size produced different capacitance signals. When using the linearized full plasmid containing the *lac* operator, the observed capacitance signal was about 3-fold higher than the one obtained for target *lac* operator (84 bp fragment with the mixture of non-target larger fragment). In the latter case, due to the smaller size of the specific operator fragment, lower capacitance changes were obtained. The results indicate that smaller DNA molecules provide a better signal for binding to the *lac* repressor. Interestingly, this is in contrast to previous results using different techniques.

Furthermore, the different sensitivities observed indicate clearly that the *lac* repressor protein modified electrode recognizes specifically the operator DNA fragment.

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What is claimed is:

1. A device comprising a solid element covered by a layer of a noble metal, wherein the layer of noble metal is covered by a layer of a monolayer-forming, self-assembling monolayer-forming molecules to which are bound a protein that has nucleic acid transcription regulatory activity.
2. The device of claim 1, wherein the solid element is plastic.
3. The device of claim 1, wherein the noble metal is gold.
4. The device of claim 1, wherein the monolayer-forming, self-assembling monolayer-forming molecules comprise at least one thiol, sulfide, or disulfide group.
5. The device of claim 4, wherein the monolayer-forming, self-assembling monolayer-forming molecules comprise thiocetic acid, cysteamine, or both.
6. The device of claim 1, wherein the layer of a monolayer-forming, self-assembling monolayer-forming molecules comprises an alkyl-thiol layer.
7. A method for detecting an interaction between a protein having nucleic acid transcription regulatory activity and a substance, said method comprising
 - providing a device comprising a solid element covered by a layer of a noble metal, wherein the layer of noble metal is covered by a monolayer of self-assembling, monolayer-forming molecules to which are bound a protein that has nucleic acid transcription regulatory activity;
 - exposing the device to a sample suspected of containing the substance under conditions where specific interaction of the substance with the protein of the device can occur; and
 - determining whether the protein has interacted with the substance by determining whether a signal is produced by the device.

8. The method of claim 7, wherein an interaction between the substance and the protein results in a conformational change in the protein.

9. The method of claim 7, wherein the substance is an inducer of activity of the protein.

10. The method of claim 7, wherein the substance is a repressor of activity of the protein.

11. The method of claim 7, wherein the substance is a drug.

12. A method for detecting the presence of a substance in a sample suspected of containing the substance, said method comprising

providing a device comprising a solid element covered by a layer of a noble metal, wherein the layer of noble metal is covered by a monolayer of self-assembling, monolayer-forming molecules to which are bound a protein that has nucleic acid transcription regulatory activity;

exposing the device to the sample suspected of containing the substance under conditions where specific interaction of the substance with the protein of the device can occur; and

determining whether the protein has interacted with the substance by determining whether a signal is produced by the device,

wherein interaction between the protein and the substance is indicated by the presence of a signal produced by the device.

13. The method of claim 12, wherein an interaction between the substance and the protein results in a conformational change in the protein.

14. The method of claim 12, wherein the substance is an inducer of activity of the protein.

15. The method of claim 12, wherein the substance is a repressor of activity of the protein.
16. The method of claim 12, wherein the substance is a drug.
17. The method of claim 12, further comprising determining the concentration of the substance in the sample.
18. A method for identifying amino acid residues of a protein having nucleic acid transcription regulatory activity that are involved in binding of the protein to at least one substance, said method comprising
- providing a device comprising a solid element covered by a layer of a noble metal, wherein the layer of noble metal is covered by a monolayer of self-assembling, monolayer-forming molecules to which are bound a protein that has nucleic acid transcription regulatory activity;
 - exposing the device to the sample suspected of containing the substance under conditions where specific interaction of the substance with the protein of the device can occur; and
 - determining whether the protein has interacted with the substance by determining whether a signal is produced by the device,
 - wherein the presence of a signal produced by the device indicates that amino acid residues that are involved in binding of the protein to said at least one substance are present in the protein.
19. The method of claim 18, wherein the protein is a fragment of a protein having nucleic acid transcription regulatory activity.

Figure 1

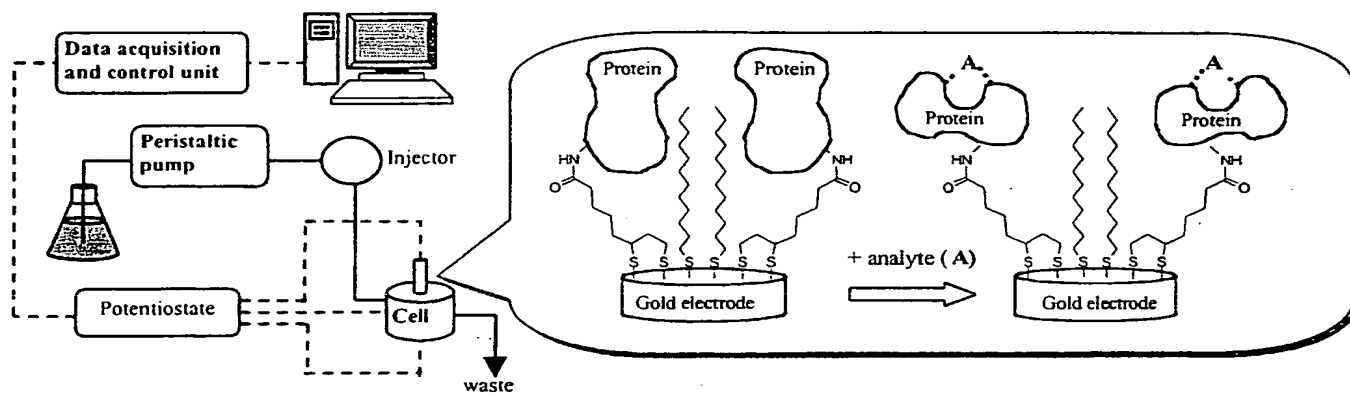


Figure 2

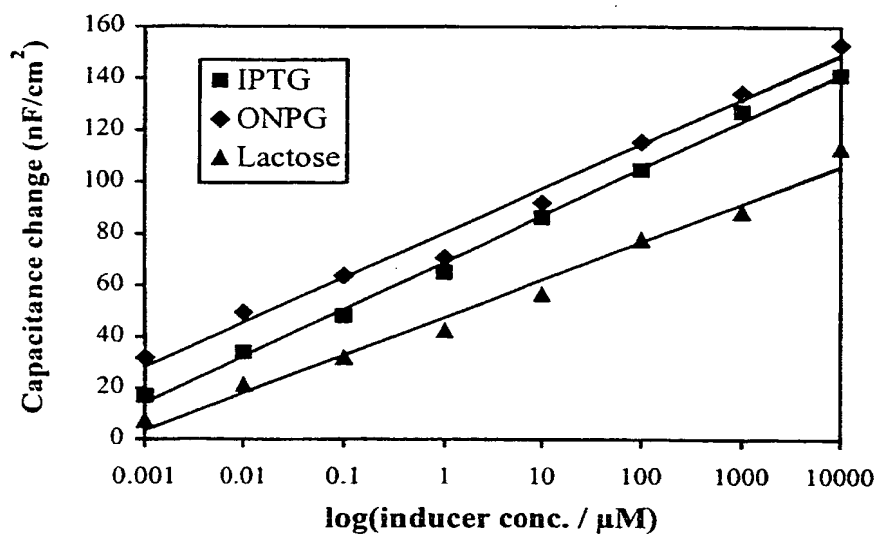
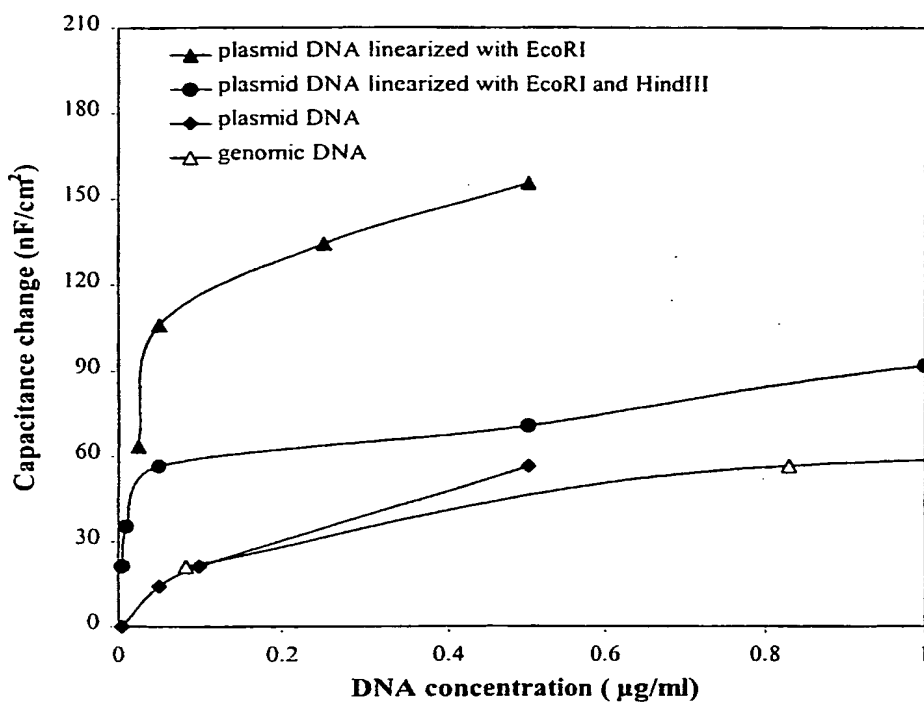


Figure 3

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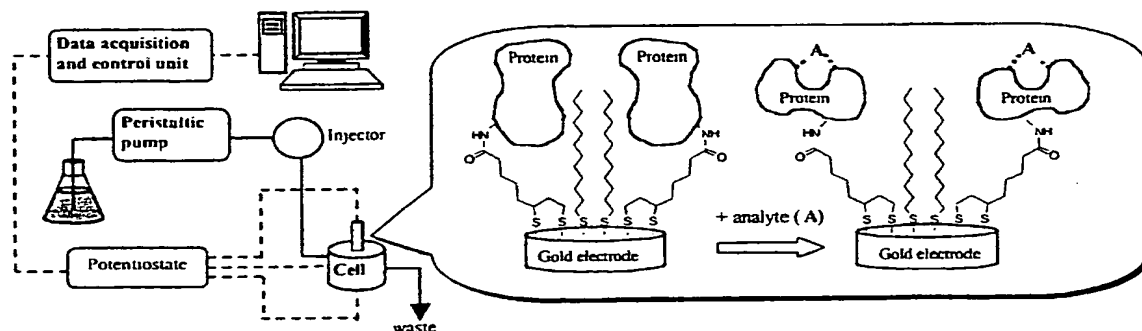
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(54) Title: DEVICE FOR MONITORING ANALYTES AT SUB-MICROMOLAR CONCENTRATIONS



(57) Abstract: The invention provides a device for detecting sub-micromolar concentrations of substances that interact with proteins having nucleic acid transcription regulatory activity. The device comprises an electrode having a surface bound by proteins having nucleic acid transcription regulatory activity. Interaction of the protein with a substance induces a conformational change in the protein, resulting in generation of a signal by the device. The invention also provides methods of detecting substances that interact with proteins having nucleic acid transcription regulatory activity as well as methods of identifying active sites of proteins having nucleic acid transcription regulatory activity.

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INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER

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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-INTERNAL, BIOSIS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 0004390 A2 (ZYOMYX, INC.), 27 January 2000 (27.01.00), the whole document, specially fig 6 and claims 35 & 43	1-7,9-12, 15-17
A	--	8,13
X	US 5620850 A (CYNTHIA C. BAMDAD ET AL), 15 April 1997 (15.04.97), the whole document, specially column 4-7 and example 5	1-8,12-13,17
A	-----	9-11,14-16

☐ Further documents are listed in the continuation of Box C.
 ☒ See patent family annex.

<p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier application or patent but published on or after the international filing date</p> <p>"I" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"F" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>
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Date of the actual completion of the international search

7 Sept 2001

Date of mailing of the international search report

10.10.01

Name and mailing address of the International Searching Authority
European Patent Office P.B. 5818 Patentlaan 2
NL-2280 HV Rijswijk
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Authorized officer

CARL-OLOF GUSTAFSSON/EÖ
Telephone No.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB01/00936

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see next sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB01/00936

I: A device comprising a solid element covered by a layer of a noble metal, wherein the noble metal is covered by a self-assembling monolayer to which is bound a protein that has nucleic acid transcription regulatory activity. Methods for detecting an interaction between a protein having nucleic acid transcription regulatory activity and a substance and for detecting the presence of a substance in a sample which interact with a protein having nucleic acid transcription regulatory activity, where the aforementioned device is used. Claims 1-17.

II: A method for identifying amino acid residues of a protein having nucleic acid transcription regulatory activity that are involved in binding of the protein to a substance, where the aforementioned device is used. Claims 18-19.

346061

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/08/01

International application No.

PCT/IB 01/00936

Patent document cited in search report			Publication date	Patent family member(s)		Publication date
WO	0004390	A2	27/01/00	AU	5102499 A	07/02/00
				EP	1097379 A	09/05/01

US	5620850	A	15/04/97	US	6197515 B	06/03/01

Form PCT/ISA 210 (patent family annex) (July 1998)

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(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
8 November 2001 (08.11.2001)

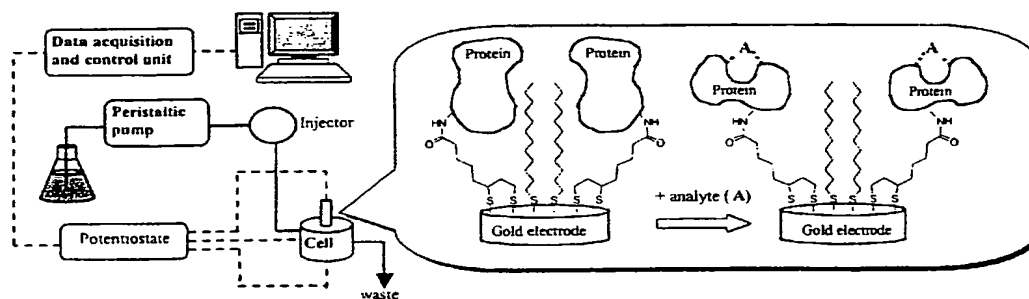
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(10) International Publication Number
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C12Q 1/25, C12M 1/40, B01L 11/00(21) International Application Number: **PCT/IB01/00936**(22) International Filing Date: **4 May 2001 (04.05.2001)**(25) Filing Language: **English**(26) Publication Language: **English**(30) Priority Data:
0001669-1 **4 May 2000 (04.05.2000) SE**(71) Applicant (for all designated States except US):
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MATTIASSON, Bo [SE/SE]; Trädgårdsmästaren 26,
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derson, Farabow, Garrett & Dunner, L.L.P., 1300 I Street,
N.W., Washington, DC 20005-3315 (US).**(81) Designated States (national): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,
CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM,
HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK,
LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX,
MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL,
TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.(84) Designated States (regional): ARIPO patent (GH, GM,
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patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,
CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).**Published:**

- with international search report
- with amended claims

(88) Date of publication of the international search report:
21 March 2002**Date of publication of the amended claims: 4 July 2002***For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.*(54) Title: **DEVICE FOR MONITORING ANALYTES AT SUB-MICROMOLAR CONCENTRATIONS**

(57) **Abstract:** The invention provides a device for detecting sub-micromolar concentrations of substances that interact with proteins having nucleic acid transcription regulatory activity. The device comprises an electrode having a surface bound by proteins having nucleic acid transcription regulatory activity. Interaction of the protein with a substance induces a conformational change in the protein, resulting in generation of a signal by the device. The invention also provides methods of detecting substances that interact with proteins having nucleic acid transcription regulatory activity as well as methods of identifying active sites of proteins having nucleic acid transcription regulatory activity.

AMENDED CLAIMS

[received by the International Bureau on 10 December 2001 (10.12.01);
new claims 20-24 added; remaining claims unchanged (1 page)]

20. The method of claim 7, wherein the signal is a change in capacitance.
21. The method of claim 12, wherein the signal is a change in capacitance.
22. The method of claim 18, wherein the signal is a change in capacitance.
23. A method for detecting an interaction between a protein that undergoes a conformational change upon binding to a substance other than a heavy metal, said method comprising
- providing a device comprising a solid element covered by a layer of a noble metal, wherein the layer of noble metal is covered by a monolayer of self-assembling, monolayer-forming molecules to which are bound a protein that undergoes a conformational change upon binding to a substance other than a heavy metal;
 - exposing the device to a sample suspected of containing the substance under conditions where specific interaction of the substance with the protein of the device can occur; and
 - determining whether the protein has interacted with the substance by determining whether a signal is produced by the device.
24. The method of claim 23, wherein the signal is a change in capacitance.